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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/DK94/00264 <b>(22) International Filing Date:</b> 28 June 1994 (28.06.94)  <b>(30) Priority Data:</b> 0767/93                      28 June 1993 (28.06.93)                      DK  <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ANDERSEN, Jens, Toenne [DK/DK]; Skyttebjerg 85, DK-2850 Nærum (DK). HJORT, Carsten, M. [DK/DK]; Gåseageren 43, DK-4000 Roskilde (DK). DIDERICHSEN, Børge [DK/DK]; Fuglesangsvej 4, DK-3460 Birkerød (DK). AASLYNG, Dorrit [DK/DK]; Fyren 8, Svogerslev, DK-4000 Roskilde (DK). HANSEN, Christian, K. [DK/DK]; Øster Farimagsgade 93,4, DK-2100 Copenhagen Ø (DK).  <b>(74) Common Representative:</b> NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).		<b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, FL, HU, JP, KE, KG, KP, KR, KZ, LK, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> A METHOD OF PRODUCING A PROTEIN DISULFIDE REDOX AGENT		
<b>(57) Abstract</b>  The invention relates to a method of producing a protein disulfide redox agent, comprising i) cloning a DNA sequence coding for said protein disulfide redox agent from a donor cell, ii) making a DNA construct wherein said DNA sequence is under control of regulatory elements, iii) introducing said DNA construct into a host cell, iv) growing said host cell under conditions conducive to the production of the protein disulfide redox agent, and v) recovering and purifying said protein disulfide redox agent. Furthermore compositions comprising (i) a protein disulfide redox agent in combination with (ii) at least one redox partner, and optionally (iii) at least one or more other enzymes are demonstrated. The compositions can be used for the treatment or degradation of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, and pharmaceuticals for the alleviation of eye sufferings.		

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## A METHOD OF PRODUCING A PROTEIN DISULFIDE REDOX AGENT

### FIELD OF THE INVENTION

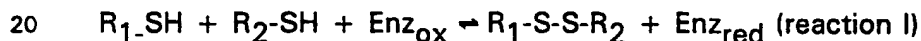
The present invention relates to a method of producing a protein disulfide redox agents, a protein disulfide redox agent product, a DNA construct encoding a  
5 protein disulfide redox agent, a vector comprising said DNA construct, and a cell comprising said vector.

The invention furthermore relates to compositions comprising (i) a protein disulfide redox agent in combination with (ii) at least one redox partner, and optionally (iii) at least one or more other enzymes. The compositions can be used  
10 for the treatment or degradation of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, and pharmaceuticals for the alleviation of eye sufferings.

### BACKGROUND OF THE INVENTION

15 The use of protein disulfide redox agents such as protein disulfide reductases, protein disulfide isomerases, protein disulfide oxidases, protein disulfide oxidoreductase, protein disulfide transhydrogenases, sulfhydryl oxidase, and thioredoxins for various purposes has been known for some time.

Protein disulfide redox agents catalyses the general reaction:



where  $R_1$  and  $R_2$  represent protein entities which are the same or different, either within the same polypeptide or in two polypeptides,  $\text{Enz}_{\text{ox}}$  is a protein disulfide redox agent in the oxidised state, and  $\text{Enz}_{\text{red}}$  is a protein disulfide redox agent in the reduced state. EC 5.3.4.1 (Enzyme Nomenclature, Academic Press, Inc.,  
25 1992) refers to an enzyme capable of catalysing the rearrangement of -S-S-

bonds in proteins and EC 1.6.4.4 and EC 1.8.4.2 is an example of enzymes catalysing the reaction with NAD(P)H and glutathione as a mediator, respectively.

This type of activity has in the past been designated as e.g. protein disulfide isomerase, protein disulfide oxidase, protein sulfhydryl oxidase, protein disulfide  
5 reductase, sulfhydryl isomerase, disulfide isomerase, protein disulfide transhydrogenase, protein disulfide oxidoreductase and sulfhydryl oxidase.

The uses of such enzymes have all been connected with reduction of protein disulfide linkages to free protein sulfhydryl groups and/or the oxidation of protein sulfhydryl groups to protein disulfide linkages, and/or the rearrangement of  
10 disulfide linkages in the same or between different polypeptides, and sometimes with both these processes in sequence.

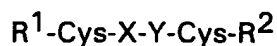
The protein disulfide redox agents of this invention can be divided into four main groups of enzymes, thioredoxin type (TRX), protein disulfide isomerase type (PDI), disulfide Bond Formation protein type (DsbA) and protein engineered derivatives,  
15 chemical modifications and hybrids of TRX and/or PDI and/or DsbA (ENG, sometimes also designated variants or muteins of TRX, PDI or DsbA).

TRX is a 12-kDa protein having a redox-active disulfide/dithiol and catalysing thiol-disulfide exchange reactions (Edman et al., Nature 317:267-270, 1985; Holmgren, Annu. Rev. Biochem. 54:237-271, 1985; Holmgren, J. Biol. Chem.  
20 264:13963-13966, 1989).

PDI consists of two subunits, each consisting of two domains which are homologous to TRX.

DsbA is a 21-kDa protein known to be capable of reducing disulfide bonds of insulin and activity common to disulfide oxidoreductases (Bardwell et al., Cell,  
25 Vol. 67, 581-589, 1991).

TRX, dsbA and the two domains in the subunits of PDI generally comprise a sequence which may be represented as below:

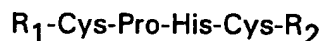


For TrX and PDI,  $R^1$  and  $R^2$  are each different amino acid sequences, X generally is Gly, and Y generally is Pro or His, respectively.

TRX from the T<sub>4</sub>-bacteriophage has the sequence:



DsbA from *E. coli* has generally the sequence:



- 10 In the context of this invention a protein disulfide redox agent may therefore be defined as an enzyme exhibiting the above sequence, but where X and Y can be any amino acid residue, and catalysing reaction I above.

ENG can be prepared by a variety of methods based on standard recombinant DNA technology:

- 15 1) by using site-directed or random mutagenesis to modify the genes encoding TRX, dsbA or PDI in order to obtain ENG with one or more amino acid changes, such as replacements, insertions, and/or deletions,  
2) by inhibiting or otherwise avoiding dimerisation of the subunits of PDI, thus giving rise to PDI monomers,  
20 3) by producing partial monomers of PDI, DsbA or TRX, in which regions of the NH<sub>2</sub>- or COOH termini of PDI, DsbA or TRX are lacking,  
4) by creating hybrids of PDI, DsbA, TRX and/or ENG,  
5) by chemically or enzymatically modifying the products of 1)-4), and  
6) by a combination of any of 1)-5).  
25 ENG preparation by standard recombination DNA technology for TRX and PDI according to 1) was described by Lundström et al. (J. Biol. Chem. 267:9047-9052, 1992) and by a combination of 3) and 5) by Pigiet (WO 8906122).

PDI, DsbA, TRX and ENG can be obtained by purification from 1) animal or 2) plant tissues, or from 3) microorganisms, or 4) by expression of recombinant DNA encoding plant, animal, human or microbial PDI, dsbA, TRX or ENG in microorganisms or other suitable hosts, followed by purification of PDI, DsbA, TRX or ENG from extracts or supernatants of said microorganisms. Preparation of TRX according to 1) was described by Luthman and Holmgren (Biochem. 121:6628-6633, 1982), according to 2) by Wada and Buchanan (in "Thioredoxins, structure and function" (Gadal, Ed.) Editions du Centre National de la Recherche Scientifique), according to 3) by Porque et al. (J. Biol. Chem. 245:2362-2379, 1970) and 10 by Laurent et al. (J. Biol. Chem. 239:3436-3445), and according to 4) by Krause et al. (J. Biol. Chem. 266:9494-9500). PDI has been prepared according to 1) by Lambert and Freedman (Biochem J. 213:225-234, 1983), according to 3) by Starnes et al. (US 4632905) and by Hammer et al. (US 4894340), and according to 4) by among others Yamauchi et al. (Biochem. Biophys. Res. Commun. 15 146:1485-1492, 1987). Finally, an ENG was prepared by Lundström et al. (J. Biol. Chem. 267:9047-9052, 1992) according to 4).

Disulfide linkages in proteins are formed between cysteine residues and have the general function of stabilising the three dimensional structure of the proteins. They can be formed between cysteine residues of the same or different 20 polypeptides.

Disulfide linkages are present in many types of proteins such as enzymes, structural proteins, etc. Enzymes are catalytic proteins such as proteases, amylases, etc., while structural proteins can be scleroproteins such as keratin, etc, protein material in hair, wool, skin, leather, hides, food, fodder, stains, and 25 human tissue contain disulfide linkages. Treatment of some of these materials with PDI and TRX, and a redox partner has been described previously.

The use of TRX for waving, straightening, removing and softening of human and animal hair was described by Pigiet et al. (EP 183506 and WO 8906122). Pigiet (US 4771036) also describes the use of TRX for prevention and reversal of 30 cataracts. Schreiber (DE 2141763 and DE 2141764) describes the use of protein

disulfide transhydrogenase for changing the form of human hair. Pigiet (EP 225156) describes the use of TRX for refolding denatured proteins. Use of TRX to prevent metal catalysed oxidative damage in biological reactions is described by Pigiet et al. (EP 237189).

5 Toyoshima et al. (EP 277563 and EP 293793) describe the use of PDI to catalyses renaturation of proteins having reduced disulfide linkages or unnatural oxidised disulfide linkages, in particular in connection with renaturation of recombinantly produced proteins. Brockway (EP 272781), and King and Brockway (EP 276547) describe the use of PDI for reconfiguration of human hair,  
10 and for treatment of wool, respectively. Sulfhydryl oxidase for the treatment of Ultra-high temperature sterilized milk is described in US 4894340, US 4632905, US 4081328 and US 4053644. Schreiber (DE 2141763 and DE 2141764) describes the use of protein disulfide transhydrogenase for changing the form of human hair.

## 15 ABBREVIATIONS

### AMINO ACIDS

	A	=	Ala	=	Alanine
	V	=	Val	=	Valine
	L	=	Leu	=	Leucine
20	I	=	Ile	=	Isoleucine
	P	=	Pro	=	Proline
	F	=	Phe	=	Phenylalanine
	W	=	Trp	=	Tryptophan
	M	=	Met	=	Methionine
25	G	=	Gly	=	Glycine
	S	=	Ser	=	Serine
	T	=	Thr	=	Threonine
	C	=	Cys	=	Cysteine
	Y	=	Tyr	=	Tyrosine
30	N	=	Asn	=	Asparagine



Q	=	Gln	=	Glutamine
D	=	Asp	=	Aspartic Acid
E	=	Glu	=	Glutamic Acid
K	=	Lys	=	Lysine
5 R	=	Arg	=	Arginine
H	=	His	=	Histidine

#### NUCLEIC ACID BASES

A	=	Adenine	
G	=	Guanine	
10 C	=	Cytosine	
T	=	Thymine	(only in DNA)
U	=	Uracil	(only in RNA)

In the Tables "deletions" are indicated by "-", e.g. "SI--AKA" indicating that for this protein it appears as if two deletions have occurred compared to the other  
15 proteins in the Tables.

#### MUTATIONS

In describing the various mutants produced or contemplated according to the invention, the following nomenclatures were adapted for ease of reference:

Original amino acid(s) position(s) substituted amino acid(s)

20 According to this the substitution of Glutamic acid for glycine in position 195 is designated as:

Gly 195 Glu or G195E

a deletion of glycine in the same position is:

Gly 195 \* or G195\*

25 and insertion of an additional amino acid residue such as lysine is:

Gly 195 GlyLys or G195GK

Where a deletion is indicated in the Tables, or present in a protein not indicated in the Tables, an insertion in such a position is indicated as:

\* 36 Asp                      or                      \*36D

for insertion of an aspartic acid in position 36

5 Multiple mutations are separated by pluses, i.e.:

Arg 170 Tyr + Gly 195 Glu   or      R170Y + G195E

representing mutations in positions 170 and 195 substituting tyrosine and glutamic acid for arginine and glycine, respectively.

#### SUMMARY OF THE INVENTION

- 10 The present invention relates a method of producing a protein disulfide redox agent, comprising
- i) cloning a DNA sequence coding for said protein disulfide redox agent from a donor cell,
  - ii) making a DNA construct wherein said DNA sequence is under control of
  - 15 regulatory elements,
  - iii) introducing said DNA construct into a host cell,
  - iv) growing said host cell under conditions conducive to the production of the protein disulfide redox agent, and
  - v) recovering and purifying said protein disulfide redox agent.
- 20 In a preferred embodiment the protein disulfide redox agent is secreted into the medium.

Further the invention relates to a protein disulfide redox agent product.

It is also the object of the invention to provide a composition of matter comprising

- (i) a protein disulfide redox agent, optionally (ii) at least a redox partner, and
- 25 optionally (iii) one or more other enzymes.

Another object of the invention is to provide processes for using said compositions, and finally a DNA construct encoding said protein disulfide redox agents, an expression vector comprising said DNA constructs, and a cell comprising said vector.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS AND TABLES

Figure 1 displays the plasmid map of pCaHj435 made from the *E. Coli* expression vector pHD 389 (Lopez - Otin et al., J. Biol. Chem., in press) comprising the dsbA gen sequence.

Figure 2 displays the plasmid map of pPL1759 (Hansen. C., Thesis, The Technical  
10 University of Denmark, 1992).

Figure 3 displays the plasmid map of pJA146 made from the pPL1759 plasmid containing the putative mature dsbA encoding region (J.C.A. Bardwell et al., Cell, 67, p. 581-589, 1991).

Table 1 shows an alignment of published eukaryotic PDI amino acid sequences:  
15 Bovine (*Bos taurus*) (Yamauchi et al., Biochem. Biophys. Res. Commun. 146:1485-1492, 1987), chicken (*Gallus gallus*) (Parkkonen et al., Biochem. J. 256:1005-1011, 1988), human (*Homo sapiens*) (Rapilajaniemi et al. EMBO J. 6:643-649, 1987), mouse (*Mus musculus*) (Gong, et al., Nucleic Acids Res. 16:1203, 1988), rabbit (*Oryctolagus cuniculus*) (Fliegel et al., J. Biol. Chem. 20 265:15496-15502, 1990), rat (*Rattus norvegicus*) (Edman et al., Nature 317:267-270, 1985), and yeast (*Saccharomyces cerevisiae*) (Tachikawa et al., J. Biochem. 110:306-313).

Table 2 shows an alignment of PDI amino acid sequences: Alfalfa (*Medicago sativa*) (Shorrosh and Dixon, Plant. Mol. Bio. 19:319-321, 1992), *A. oryzae*,  
25 yeast (*Saccharomyces cerevisiae*) (Tachikawa et al., J. Biochem. 110:306-313), bovine (*Bos taurus*) (Yamauchi et al., Biochem. Biophys. Res. Commun.

146:1485-1492, 1987), rat (*Rattus norvegicus*) (Edman et al., *Nature* 317:267-270, 1985), and mouse (*Mus musculus*) (Gong, et al., *Nucleic Acids Res.* 16:1203, 1988).

#### DETAILED DESCRIPTION OF THE INVENTION

- 5 The object of the invention is to provide a method of producing a protein disulfide redox agent, comprising
- i) cloning a DNA sequence coding for said protein disulfide redox agent from a donor cell,
  - ii) making a DNA construct wherein said DNA sequence is under control of
  - 10 regulatory elements,
  - iii) introducing said DNA construct into a host cell,
  - iv) growing said host cell under conditions conducive to the production of the protein disulfide redox agent, and
  - v) recovering and purifying said protein disulfide redox agent.
- 15 In a preferred embodiment of the invention the protein disulfide redox agent is secreted into the medium.

In another embodiment the DNA construct is introduced into a host cell of a species different from the donor cell,

In an embodiment of the method according to the invention the protein disulfide  
20 redox agent is expressed in the form of a proenzyme and the cell is cultured in the presence of a proteolytic enzyme capable of converting the proenzyme of the protein disulfide redox agent into a mature enzyme.

Preferably, said donor and host cells are microbial, either bacterial cells or a fungal cells.

In an embodiment both said donor and host cells are bacterial. In a preferably embodiment said bacterial cells is gram-positive and one is gram-negative.

In another embodiment both said donor and host cells are fungal.

In still an embodiment of the invention one of said microbial cells is bacterial and  
5 one is fungal cell.

According to the invention the bacterial cell is a cell of a gram-positive bacterium, e.g. of the genus *Bacillus* or *Streptomyces* or a cell of a gram-negative bacterium, e.g. of the genus *Escherichia*, and the fungal cell is a yeast cell, e.g. of the genus *Saccharomyces*, or a cell of a filamentous fungus, e.g. of the genus *Aspergillus*  
10 or *Fusarium*.

In a preferred embodiment said *Escherichia* is *E. coli*, said *Aspergillus* is *Aspergillus niger*, *Aspergillus oryzae*, or *Aspergillus nidulans*, and said *Bacillus* is *Bacillus licheniformis*, *Bacillus lentus*, or *Bacillus subtilis*.

Many methods for introducing mutations into genes are well known in the art.  
15 After a brief discussion of cloning protein disulfide redox agent genes, methods for generating mutations in both random sites, and specific sites, within the protein disulfide redox agent gene will be discussed.

#### Cloning a protein disulfide redox agent gene

The DNA sequence of the DNA construct of the invention may be isolated by  
20 well-known methods. Thus, the DNA sequence may, for instance, be isolated by establishing a cDNA or genomic library from an organism expected to harbour the sequence, and screening for positive clones by conventional procedures. Examples of such procedures are hybridization to oligonucleotide probes synthesized on the basis of any of the full amino acid sequences shown in Tables 1  
25 and 2, or a subsequence thereof in accordance with standard techniques (cf. Sambrook et al., 1989), and/or selection for clones expressing a protein disulfide redox agent activity as defined above, and/or selection for clones producing a

protein which is reactive with an antibody raised against the protein disulfide redox agent comprising any of the amino acid sequences shown in Tables 1 and 2.

A preferred method of isolating a DNA construct of the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the amino acid sequence of the parent protein disulfide redox agent of the invention. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202 or by R.K. Saiki et al. (1988).

10 Alternatively, the DNA sequence of the DNA construct of the invention may be prepared synthetically by established standard methods, e.g. the phosphoramidite method described by Beaucage and Caruthers (1981), or the method described by Matthes et al. (1984). According to the phosphoramidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, 15 ligated and cloned in appropriate vectors.

Finally, the DNA construct may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire recombinant DNA molecule, in accordance with standard techniques. 20

#### Generation of random mutations in the protein disulfide redox agent gene

Once the protein disulfide redox agent gene has been cloned into a suitable vector, such as a plasmid, several methods can be used to introduce random mutations into the gene.

25 One method would be to incorporate the cloned protein disulfide redox agent gene, as part of a retrievable vector, into a mutator strain of *Eschericia coli*.

Another method would involve generating a single stranded form of the protein disulfide redox agent gene, and then annealing the fragment of DNA containing the protein disulfide redox agent gene with another DNA fragment such that a portion of the protein disulfide redox agent gene remained single stranded. This discrete, single stranded region could then be exposed to any of a number of mutagenizing agents, including, but not limited to, sodium bisulfite, hydroxylamine, nitrous acid, formic acid, or hydralazine. A specific example of this method for generating random mutations is described by Shortle and Nathans (1978, Proc. Natl. Acad. Sci. U.S.A., 75: 2170-2174). According to the Shortle and Nathans method, the plasmid bearing the protein disulfide redox agent gene would be nicked by a restriction enzyme that cleaves within the gene. This nick would be widened into a gap using the exonuclease action of DNA polymerase I. The resulting single-stranded gap could then be mutagenized using any one of the above mentioned mutagenizing agents.

15 Generation of site directed mutations in the protein disulfide redox agent gene

Once the protein disulfide redox agent gene has been cloned, and desirable sites for mutation identified, these mutations can be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites; mutant nucleotides are inserted during oligonucleotide synthesis. In a preferred method, a single stranded gap of DNA, bridging the protein disulfide redox agent gene, is created in a vector bearing the protein disulfide redox agent gene. Then the synthetic nucleotide, bearing the desired mutation, is annealed to a homologous portion of the single-stranded DNA. The remaining gap is then filled in by DNA polymerase I (Klenow fragment) and the construct is ligated using T4 ligase. A specific example of this method is described in Morinaga et al., (1984, Biotechnology 2:646-639). According to Morinaga et al., a fragment within the gene is removed using restriction endonuclease. The vector/gene, now containing a gap, is then denatured and hybridized to a vector/gene which, instead of containing a gap, has been cleaved with another restriction endonuclease at a site outside the area involved in the gap. A single-stranded region of the gene is then available for hybridization with mutated oligonucleotides, the remaining gap is filled in by the Klenow fragment

of DNA polymerase I, the insertions are ligated with T4 DNA ligase, and, after one cycle of replication, a double-stranded plasmid bearing the desired mutation is produced. The Morinaga method obviates the additional manipulation of constructing new restriction sites, and therefore facilitates the generation of 5 mutations at multiple sites.

#### Expression of protein disulfide redox agent

According to the invention, a protein disulfide redox agent gene can be expressed, in enzyme form, using an expression vector. An expression vector generally falls under the definition of a cloning vector, since an expression vector 10 usually includes the components of a typical cloning vector, namely, an element that permits autonomous replication of the vector in a microorganism independent of the genome of the microorganism, and one or more phenotypic markers for selection purposes. An expression vector includes control sequences encoding a promoter, operator, ribosome binding site, translation initiation signal, and, 15 optionally, a repressor gene or various activator genes. To permit the secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the coding sequence of the gene. For expression under the direction of control sequences, a target gene to be treated according to the invention is operably linked to the control sequences in the proper reading frame. 20 Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant protein disulfide redox agent gene, include but are not limited to the prokaryotic  $\beta$ -lactamase promoter (Villa-Komaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references 25 can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

The expression vector carrying the DNA construct of the invention may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be 30 introduced. Thus, the vector may be an autonomously replicating vector, i.e. a



vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid, a bacteriophage or an extrachromosomal element, minichromosome or an artificial chromosome. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA construct of the invention, especially in a bacterial host, are the promoter of the *lac* operon of *E.coli*, the *Streptomyces coelicolor* agarase gene *dagA* promoters, the promoters of the *Bacillus licheniformis*  $\alpha$ -amylase gene (*amyL*), the promoters of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the promoters of the *Bacillus Amyloliquefaciens*  $\alpha$ -amylase (*amyQ*), the promoters of the *Bacillus subtilis* *xylA* and *xylB* genes etc. For transcription in a fungal host, examples of useful promoters are those derived from the gene encoding *A. oryzae* TKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral  $\alpha$ -amylase, *A. niger* acid stable  $\alpha$ -amylase, *A. niger* glucoamylase, *Rhizomucor miehei* lipase, *A. oryzae* alkaline protease, *A. oryzae* triose phosphate isomerase or *A. nidulans* acetamidase.

The expression vector of the invention may also comprise a suitable transcription terminator and, in eukaryotes, polyadenylation sequences operably connected to the DNA sequence encoding the protein disulfide redox agent of the invention. Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. Examples of such sequences are the origins of repli-

cation of plasmids pUC19, pACYC177, pUB110, pE194, pAMB1, pHD 389 and pIJ702.

The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the *dal* genes from  
5 *B.subtilis* or *B.licheniformis*, or one which confers antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracyclin resistance. Examples of *Aspergillus* selection markers include *amdS*, *argB*, *niaD* and *sC*, a marker giving rise to hygromycin resistance. Furthermore, the selection may be accomplished by co-transformation, e.g. as described in WO 91/17243.

10 While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. The protein disulfide redox agents of the invention comprising a variant of any of the amino acid sequences shown in tables 1 or 2 may furthermore comprise a preregion permitting secretion of the expressed protein disulfide  
15 isomerase into the culture medium. If desirable, this preregion may be native to the protein disulfide isomerase of the invention or substituted with a different preregion or signal sequence, conveniently accomplished by substitution of the DNA sequences encoding the respective preregions.

The procedures used to ligate the DNA construct of the invention, the promoter,  
20 terminator and other elements, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al. (1989)).

The cell of the invention either comprising a DNA construct or an expression vector of the invention as defined above is advantageously used as a host cell in  
25 the recombinant production of a polypeptide of the invention. The cell may be transformed with the DNA construct of the invention, conveniently by integrating the DNA construct in the host chromosome. This integration is generally considered to be an advantage as the DNA sequence is more likely to be stably maintained in the cell. Integration of the DNA constructs into the host chromosome

may be performed according to conventional methods, e.g. by homologous or heterologous recombination. Alternatively, the cell may be transformed with an expression vector as described above in connection with the different types of host cells.

- 5 The cell of the invention may be a cell of a higher organism such as a mammal, an avian, an insect, or a plant cell, but is preferably a microbial cell, e.g. a bacterial or a fungal (including yeast) cell.

Examples of suitable bacteria are gram positive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*,  
10 *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus*, *Bacillus megaterium*, *Bacillus thuringiensis*, or *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E.coli*. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se*.

- 15 The yeast organism may favourably be selected from a species of *Saccharomyces* or *Schizosaccharomyces*, e.g. *Saccharomyces cerevisiae*. The filamentous fungus may advantageously belong to a species of *Aspergillus*, e.g. *Aspergillus oryzae* or *Aspergillus niger*. Alternatively, a strain of a *Fusarium* species, e.g. *F. oxysporum*, can be used as a host cell. Fungal cells may be transformed by a process  
20 involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known *per se*. A suitable procedure for transformation of *Aspergillus* host cells is described in EP 238 023. A suitable method of transforming *Fusarium* species is described by Malardier et al., 1989.

According to the invention expression of the DNA construct comprising the DNA  
25 sequence or expression vector carrying the DNA construct may take place as heterologous expression in a host cell different from the donor cell from where the DNA was derived.

According to the invention expression of prokaryote DNA may take place heterologously in cell compartments.

In a preferred embodiment according to the invention the DNA derived from a cell e.g. of the genus *Escherichia* can be expressed in an other cell e.g. of the genus  
5 *Bacillus* or *Streptomyces*.

In another preferred embodiment of the invention the DNA derived from a cell e.g. of the genus *Aspergillus* can be expressed in a cell e.g. of the genus *Bacillus* or *Streptomyces*.

In a yet further aspect, the present invention relates to a method of producing a  
10 protein disulfide redox agent of the invention, which method comprises cultivating a host cell as described above under conditions conducive to the production of the protein disulfide redox agent and recovering the protein disulfide redox agent from the cells and/or culture medium.

In a specific embodiment of the invention a protein disulfide redox agent is  
15 secreted into the medium.

The medium used to cultivate the cells may be any conventional medium suitable for growing the host cell in question and obtaining expression of the protein disulfide redox agent of the invention. Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in cata-  
20 logues of the American Type Culture Collection).

The resulting protein disulfide redox agent may be recovered from the medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, if necessary after disruption of the cells, precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g.  
25 ammonium sulphate, followed by purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

It is of course also possible to produce the protein disulfide redox agents of the invention by culturing the filamentous fungal natural host or parent organism of interest and recovering the protein disulfide isomerase from the culture broth in traditional ways.

- 5 The present invention also relates to compositions comprising the protein disulfide redox agents produced according to the invention.

The compositions may suitably contain 0.01-200 mg of enzyme protein per gram, preferably 0.01-20 mg of enzyme protein per gram, especially 0.01-2 mg of enzyme protein per gram, or alternatively 0.02-0.2 mg of enzyme protein per  
10 gram, or 0.01-0.2 mg of enzyme protein per gram.

In another preferred alternative the composition contain 0.01-0.5 mg of enzyme protein per gram or alternatively 0.2-0.5 mg of enzyme protein per gram.

The compositions of the invention usually also comprises (ii) a suitable redox partner.

- 15 The redox partner (ii) is generally an organic or inorganic reductant, and would often be selected from the organic reductants, such as from the group comprising glutathione, L-cysteine, dithiothreitol, 2-mercaptoethanol, thioglycolic acid, L-cysteine ethylester,  $\beta$ -mercaptoethylamine, mercaptosuccinic acid,  $\beta$ -mercapto-propionic acid, dimercapto adipic acid, thiomalic acid, thioglycolamides, glycol  
20 thioglycolate, glycerol thioglycolate, thiolactic acid and salts thereof.

Among inorganic reductants sulfite and bisulfite compounds will often be preferred.

Furthermore the compositions of the invention may optionally comprise (iii) another enzyme, where said other enzyme preferably is selected among  
25 proteases, lipases, amylases, transglutaminases, or another protein disulfide redox agent

Under this aspect the invention is meant to comprise compositions comprising all types of protein disulfide redox agents including naturally occurring TRX or PDI either without or in combination with a redox partner. All types of ENG are naturally encompassed by the present invention also under this aspect.

- 5 The compositions of the invention may contain other ingredients known in the art as e.g. excipients, stabilizers, fillers, detergents, etc.

The compositions of the invention may be formulated in any convenient form, e.g. as a powder, paste, liquid or in granular form. The enzyme may be stabilized in a liquid by inclusion of enzyme stabilizers. Usually, the pH of a solution of the  
10 composition of the invention will be 5-10 and in some instances 7.0-8.5. Other enzymes such as proteases, cellulases, oxidases, peroxidases, amylases or lipases may be included in the compositions of the invention, either separately or in a combined additive.

The compositions of the invention can be used for the treatment or degradation  
15 of scleroproteins, especially hair, skin and wool, treatment and cleaning of fabrics, as additives to detergents, thickening and gelation of food and fodder, strengthening of gluten in bakery or pastry products, and as pharmaceuticals for the alleviation of eye sufferings.

The present invention is further illustrated in the following examples which should  
20 not, in any manner, be considered to limit the scope of the present invention.

## MATERIALS AND METHODS

### Strain:

*E. coli* WA803 (Maniatis et al., 1982, Molecular cloning, a laboratory manual, Cold Spring Harbor Laboratory, New York)

- 25 *B. Subtilis* DN1885 (P. L. Joergensen et al., Gene, 96, p. 37-41, 1990)  
JA146 : *B. subtilis* DN1885 harbouring the pJA146 plasmid

CaHj435 : *E. coli* harbouring the pCaHj435 plasmid

Plasmids:

pCaHj435: Figure 1, plasmid comprising the *dsbA* gene sequence in *E. Coli*  
5 expression vector pHD389.

pJA146: Figure 3, plasmid comprising the putative *dsbA* encoding region (J.C.A. Bardwell et al. Cell, 67, p. 581-589, 1991) in *B. subtilis* expression vector pPL1759 .

pPL1759: *B. subtilis* expression vector (Hansen C., 1992, Thesis, The Technical  
10 University of Denmark), figure 2.

pHD389: *E.coli* expression vector, (Lopez - Otin et al., J. Biol. Chem., in press)

Materials:

Perkin Elmer- cetus Amplitaq™ Taq polymerase

DNA sequencing kit Sequenase™ (United States Biochemicals)

15 Super Taq™ DNA polymerase/PCR buffer, HT Biotechnology Ltd.)

Terrific Broth medium (Maniatis et. al (1982) Supra)

Terrific Yeast medium (PCT/DK90/00332)

LB agar (Luria-Bertani medium/agar, C.R. Harwood and S.M. Cutting (Ed.)

Molecular Biological Methods for Bacillus, 1990, John Wiley & Sons Ltd.)

20 DEAS Sphadex A-50 column (Pharmacia Fine Chemicals AB)

Methods:

Trypsin inhibitor assay (Available from Novo Nordisk A/S)

N-terminal amino acid sequence analysis

N-terminal amino acid sequence analysis of recombinant *dsbA* was carried out  
25 following electroblotting using an Applied Biosystems 473A protein sequencer  
operated according to the manufacturers instructions.

EXAMPLES

Example 1Construction of the dsbA expression plasmid for expression in *E. coli*

The *E. coli* dsbA gene sequence was collected from the GenBank database (accession number M77746). Based on this sequence a PCR primer containing the  
5 restriction enzyme Cla I recognition sequence and 23 bases of the dsbA 5' coding sequence (primer 5513) and a PCR primer containing the restriction enzyme Sall recognition sequence and 23 bases complementary to the dsbA 3' coding sequence (primer 5512) were constructed.

5513	5' CCATCGATGAAAAAGATTTGGCTGGCGCT	3'
10 5512	5' CCGTCGACTTATTTTTTCTCGGACAGATATT	3'

Total DNA was extracted from *E. coli* strain WA803 using standard procedures.

This DNA was used without further modification as template in a PCR reaction (20 cycles) using the primers 5513 and 5512 and the Perkin Elmer- cetus Amplitaq™ Taq polymerase following the manufacturer's instructions.

15 A PCR fragment corresponding to the size of the dsbA gene was recovered from an agarose gel and digested with the restriction enzymes ClaI and Sall.

The *E. coli* expression vector pHD 389 was digested with the same enzymes, and the large vector fragment was ligated to the digested PCR fragment. The ligation mixture was used to transform *E. coli* strain WA803. After 24 hours of growth  
20 at 30°C using ampicillin selection a transformant was selected and subsequent DNA sequence analysis using the DNA sequencing kit Sequenase™ showed that a sequence identical to the published dsbA gene sequence was integrated between the lambda PR promoter and the fd terminator. This plasmid was termed pCaHj 435, and the *E. coli* strain harbouring the plasmid was termed CaHj 435.

25 A plasmid map of pCaHj 435 is shown in figure 1.



Expression of dsbA in *E. coli* phage lambda

The dsbA gene is under control of the promoter PR from the *E. coli* phage lambda. PR is repressed by CI repressor also harboured by the plasmid pCaHj 435. However the CI repressor allele used in this plasmid is temperature sensitive  
5 being active at 30°C but inactive at 42°C. Thus the dsbA gene is repressed at 30°C but expressed at 42°C.

In order to express the dsbA gene the strain CaHj 435 was grown in shake flasks containing the medium Terrific Broth at 30°C and 200 rpm until OD600 reached 0.2. Then the shake flasks were transferred to 42°C (200 rpm) for 18 hours.

10 Recovery and purification of the dsbA gene product.

1 liter of cell suspension was chilled on ice and then the periplasmic fraction of the cells was isolated by osmotic shock:

The cells were isolated by centrifugation (2500 x g, 15 min.) and resuspended in 100 ml 20% (W/V) sucrose buffered with 10 mM Tris/HCl pH 7.0. EDTA were  
15 added to a final concentration of 15 mM. The cell suspension was incubated on ice for 15 min. and then the cells were collected by centrifugation (2500 x g, 15 min.). The cells were resuspended in 70 ml of water by vigorous shaking and subsequently incubated on ice (10 min). The suspension was centrifuged (2500 x g, 15 min.) and the supernatant containing the soluble periplasmic fraction was  
20 isolated. Tris/HCl pH 7.0 was added to a final concentration of 5 mM.

The dsbA gene product was then purified by DEAE anion exchange chromatography.

A column containing 20 ml of DEAE Sephadex A-50 purchased from Pharmacia Fine Chemicals AB was equilibrated with 10 mM Tris/HCl pH 7.0. The osmotic  
25 shock preparation was applied to the column, and then the column was washed with 200 ml 10 mM Tris/HCl pH 7.0. The dsbA gene product was eluted with 50 ml 50 mM NaCl, 10 mM Tris/HCl pH 7.0. SDS polyacrylamide gel electrophoresis

showed that more than 90% of the protein isolated corresponded to the size of the dsbA gene.

Using the trypsin inhibitor assay it was shown that the purified protein has disulphide isomerase activity.

## 5 Example 2

### Construction of the dsbA expression plasmid for expression in *Bacillus*.

The *E. coli* dsbA gene sequence was collected from the GenBank database (accession number M77746). Based on the dsbA sequence from GenBank and pCaHj435 (the dsbA expression plasmid in *E. coli* (WA803)) a PCR primer  
10 containing the restriction enzyme NsiI recognition sequence and 27 bases of the dsbA 5' sequence encoding the putative N-terminal (J.C.A. Bardwell et al. Cell, 67, p. 581-589, 1991) of the mature DsbA protein (primer 5965) and a primer containing a restriction enzyme EcoRI recognition sequence and 20 bases complementary to the dsbA 3' sequence of pCaHj435 (primer 5966) was made:

15 5965 5'-CCTCATTATGCATCAGCGGCGGCGCAGTATGAAGATGGTAAACAG-3'  
5966 5'-GCGAATTCGTCTGACTTATTTTTTCTCGG-3'

A reisolated colony of WA803/pCaHj435 grown 18 hours at 30°C on LB agar plates containing 100 µg/ml ampicillin, 10 mM potassium phosphate pH 7,0 and 0,4% glucose was resuspended in 10 µl 1 x PCR buffer (Super Taq™ DNA  
20 polymerase) heated to 99°C for 5 minutes, spunned 20 000 x g for 2 min.

5 µl of this supernatant was used as template in a PCR reaction (20 cycles) using the primers 5965 and 5966 and Super Taq™ DNA polymerase following the manufators instructions.

A PCR fragment corresponding to the expected size of dsbA was recovered from  
25 an agarose gel and digested with the restriction enzymes EcoRI and PstI.

The plasmid pPL1759, fig. 2, was digested with the restriction enzymes PstI-EcoRI and the large vector fragment was ligated to the PCR fragment. Ligation mixture was transformed into *Bacillus subtilis* DN1885. Selection for transformants and reisolation of those was performed on LB medium containing  
5 10 µg Kanamycin/ml, 10 mM potassium phosphate pH 7,0, and 0,4% glucose.

DNA analysis of the plasmids from those cells using a DNA sequencing Kit (Sequenase™) showed the expected sequence of the promoter and signal peptide encoding regions of amyL (P.L. Joergensen et al., Gene, 96, p. 37-41, 1990) fused to the above mentioned putative mature dsbA encoding region. This  
10 plasmid was termed pJA146 and a *B. subtilis* DN1885 strain harbouring this plasmid was termed JA146. A plasmid map of pJA146 is shown in fig. 3.

#### Expression of dsbA in *Bacillus*

Strain JA146 was grown for 18 hours in Terrific Yeast medium at 37°C with 10 µg/ml Kanamycin and 0.4% glucose in 20 ml M-tubes at 280 rpm. Cells were  
15 harvested at 15 000 x g for 10 minutes and the supernatant was analyzed for DsbA protein. SDS-PAGE of the supernatant showed that a protein of the size of mature DsbA protein was secreted into the media. Using the trypsin inhibitor assay it was shown that the DsbA protein has disulphide isomerase activity.

The N-terminal amino acid sequence was analyzed as described above. The N-  
20 terminal amino acid of DsbA determined was : Ala-Ala-Gln-Tyr-Glu-Asp-Gly-Lys-Gln-

#### Example 3

##### The effect of waving composition on hair

Testing of the P34H variant of Thioredoxin from *E. coli* for enzymatic waving of  
25 hair.

A tress of washed human Scandinavian hair (1 gram) was wetted with water and tightly wound on a curling roller. 1 ml of a solution with the following composition and a temperature of 30°C was applied to the tress:

4 mg/ml P34H Thioredoxin

5 50 mM Phosphate buffer pH 7.5

1 mM Reduced Gluthation (Sigma)

The tress was put in a plastic bag and incubated for 60 minutes at 30°C. Then the roller was removed and the hair was rinsed with water, dried with a cotton towel, combed and air dried.

- 10 Other tresses of hair was treated like above but without addition of the P34H Thioredoxin variant.

Treatment with the P34H variant of Thioredoxin gives a significant waving effect on the hair.

#### Example 4

- 15 Waving effect on hair treated with DsbA

The following experiments compare the effect of waving on hair treated with DsbA from *E.coli* and bovine PDI.

Tresses of washed brown and fair human European hair (1 gram) was wetted with water and tightly wound on curling rollers. 1 ml of waving solution with the  
20 following composition and temperature of 30°C was applied to the tresses.

0.38 mg/ml DsbA

10mM Tris buffer pH 7.0 with 50mM NaCl

1 ml and 10 mM Reduced Gluthation (Sigma)

Tresses were put in plastic bags and stored for 60 minutes at 30°C. The hair was rinsed with water, dried with cotton towel, the rollers was removed, the hair was air dried and combed.

The results of the experiments are displayed in table A.

5 TABLE A

Hair	DsbA mg/ml	$L_{after}/L_{before}$
Brown	0	0.92
	0.38	0.73
Fair	0	0.93
	0.38	0.83

The lenght of all tresses were measured before ( $L_{before}$ ) and after ( $L_{after}$ ) treatment.

10  $L_{after}/L_{before}$  is a measurement for the waving effect on the hair.

### Example 5

#### Control of permanent waving

To control that the enzymatic waving was permanent and not only temporary the hair tresses were washed with a mild commercial shampoo.

15 After being treated, as described in example 4 and rinsed, the hair tresses were removed from the curling rollers and dried. Then control tresses and enzyme treated hair tresses were washed in either water or a mild shampoo, rinsed and air dried.

The results of the test are displayed in table B.

TABLE B

DsbA mg/ml	$L_{after}/L_{before}$ before shampooing	$L_{after}/L_{before}$ after shampooing
0	0.92	0.92
5 0.38	0.73	0.73

$L_{after}/L_{before}$  is a measurement for the waving effect on the hair.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

## (i) APPLICANT:

- (A) NAME: NOVO NORDISK A/S
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: Denmark
- (F) POSTAL CODE (ZIP): DK-2880
- (G) TELEPHONE: +45 4444 8888
- (H) TELEFAX: +45 4449 3256

- (ii) TITLE OF INVENTION: A method of producing a protein disulfide redox agent

## (iii) NUMBER OF SEQUENCES: 4

## (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30B (EPO)

## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer"

## (iii) HYPOTHETICAL: YES

## (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCGTCGACTT ATTTTTCTC GGACAGATAT T

## (2) INFORMATION FOR SEQ ID NO: 2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer"

## (iii) HYPOTHETICAL: YES

## (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CCATCGATGA AAAAGATTTG GCTGGCGCT

29

## (2) INFORMATION FOR SEQ ID NO: 3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "Primer"

## (iii) HYPOTHETICAL: YES

## (iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCTCATTATG CATCAGCGGC GCGCAGTAT GAAGATGGTA AACAG 45

## (2) INFORMATION FOR SEQ ID NO: 4:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single



(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(A) DESCRIPTION: /desc = "Primer"

(iii) HYPOTHETICAL: YES

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCGAATTCGT CGACTTATTT TTTCTCGG

Table 1:

	1				50
Pdi_Mouse	.....MLS	RALLCLALAW	AARVGADALE	EEDNVLVLKK	SNFEEALAAH
Pdi_Rat	.....MLS	RALLCLALAW	AARVGADALE	EEDNVLVLKK	SNFAEALAAH
Pdi_Bovin	.....MLR	RALLCLALTA	LFRAGAGAPD	EEDHVLVLHK	GNFDEALAAH
Pdi_Human	.....MLR	RALLCLAVAA	LVR..ADAPE	EEDHVLVLRK	SNFAEALAAH
Pdi_Rabbit	.....MLR	RAVLCLALAV	TA.GWAWAAE	EEDNVLVLKS	SNFAEELAAH
Pdi_Chick	.....	.....	.....EPL	EEDGVLVLRA	ANFEQALAAH
Pdi_Yeast	MKFSAGAVLS	WSSLLLASSV	FAQQEAVAPE	DSA.VVKLAT	DSFNEYIQSH
	51				100
Pdi_Mouse	KYLLVEFYAP	WCGHCKALAP	EYAKRAAKLK	AEGSEIRLAK	VDATEESDLA
Pdi_Rat	NYLLVEFYAP	WCGHCKALAP	EYAKAAAKLK	AEGSEIRLAK	VDATEESDLA
Pdi_Bovin	KYLLVEFYAP	WCGHCKALAP	EYAKAAGKLK	AEGSEIRLAK	VDATEESDLA
Pdi_Human	KYLLVEFYAP	WCGHCKALAP	EYAKAAGKLK	AEGSEIRLAK	VDATEESDLA
Pdi_Rabbit	KHLLVEFYAP	WCGHCKALAP	EYAKAAGKLK	AEGSDIRLAK	VDATEESDLA
Pdi_Chick	RHLLVEFYAP	WCGHCKALAP	EYAKAAAQLK	AEGSEIRLAK	VDATEEAELA
Pdi_Yeast	DLVLAEFFAP	WCGHCKNMAP	EYVKAETL.	.VEKNITLAQ	IDCTENQDLC
	101				150
Pdi_Mouse	QQYGVRGYPT	IKFFKNGDTA	SPKEYTAGRE	ADDIVNWLKK	RTGPAATTLS
Pdi_Rat	QQYGVRGYPT	IKFFKNGDTA	SPKEYTAGRE	ADDIVNWLKK	RTGPAATTLS
Pdi_Bovin	QQYGVRGYPT	IKFFKNGDTA	SPKEYTAGRE	ADDIVNWLKK	RTGPAASTLS
Pdi_Human	QQYGVRGYPT	IKFFRNGDTA	SPKEYTAGRE	ADDIVNWLKK	RTGPAATTLR
Pdi_Rabbit	QQYGVRGYPT	IKFFKNGDTA	SPKEYTAGRE	ADDIVNWLKK	RTGPAATTLA
Pdi_Chick	QQFGVRGYPT	IKFFRNGDKA	APREYTAGRE	ADDIVSWLKK	RTGPAATTLT
Pdi_Yeast	MEHNIPGFPS	LKIFKNSDVN	NSIDYEGPRT	AEAIVQFMK	QSQPAVAVVA
	151				200
Pdi_Mouse	DTAAAESLVD	SSEVTVIGFF	KOVESDSAKQ	FLLAAEAIDD	IPFGITSNSG
Pdi_Rat	DTAAAESLVD	SSEVTVIGFF	KDAGSDSAKQ	FLLAAEAVDD	IPFGITSNSD
Pdi_Bovin	DGAAAEALVE	SSEVAVIGFF	KDMESDSAKQ	FLLAAEVIDD	IPFGITSNSD
Pdi_Human	DGAAAESLVE	SSEVAVIGFF	KOVESDSAKQ	FLQAAEAIDD	IPFGITSNSD
Pdi_Rabbit	DSAAAESLVE	SSEVAVIGFF	KOVESDAKQ	FLLAAEATDD	IPFGLTASSD
Pdi_Chick	DAAAETLVD	SSEVVVIGFF	KDVTSDAAKE	FLLAAESVDD	IPFGISSAD
Pdi_Yeast	DLPAYLANET	FVTPVIVQSG	KIDADFNATF	YSMANKHFND	YDFVSAENAD
	201				250
Pdi_Mouse	VFSKYQLDKD	GVVLFKKFDE	GR..NNFEGE	ITKEKLLD.F	IKHNQLPLVI
Pdi_Rat	VFSKYQLDKD	GVVLFKKFDE	GR..NNFEGE	ITKEKLLD.F	IKHNQLPLVI
Pdi_Bovin	VFSKYQLDKD	GVVLFKKFDE	GR..NNFEGE	VTKEKLLD.F	IKHNQLPLVI
Pdi_Human	VFSKYQLDKD	GVVLFKKFDE	GR..NNFEGE	VTKENLLD.F	IKHNQLPLVI
Pdi_Rabbit	VFSRYQVHQD	GVVLFKKFDE	GR..NNFEGE	VTKEKLLD.F	IKHNQLPLVI
Pdi_Chick	VFSKYQLSQD	GVVLFKKFDE	GR..NNFEGD	LTKDNLLN.F	IKSNQLPLVI
Pdi_Yeast	..DDFKL...	SIYLPAMDE	PVYNGKKAD	IADADVFEKW	LQVEALPYFG
	251				300
Pdi_Mouse	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	DGKLSSFKRA	AEGF..KGKI
Pdi_Rat	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	DGKL SNFKKA	AEGF..KGKI
Pdi_Bovin	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	EGKL SNFKKA	AESF..KGKI
Pdi_Human	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	DGKL SNFKTA	AESF..KGKI
Pdi_Rabbit	EFTEQTAPKI	FGGEIKTHIL	LFLPRSAADH	DGKL SGFKQA	AEGF..KGKI
Pdi_Chick	EFTEQTAPKI	FGGEIKTHIL	LFLPKSVSDY	EGKLDNFKTA	AGNF..KGKI
Pdi_Yeast	EIDGSVFAQY	VESGLPLGYL	FY.....ND	EELEEYKPL	FTELAKNRC

table 1 (continued)

	301		350
Pdi_Mouse	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Rat	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Bovin	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Human	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Rabit	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Chick	LFIFIDSHT DNQRILEFFG LKKEECPAVR LITLEEEM..	.....TKY	
Pdi_Yeast	LMNFVSIDAR KFGRHAGNLN M.KEQFPLFA IHDMTEDLKY	GLPQLSEEAF	
	351		400
Pdi_Mouse	KPESDELTAE K..ITEFCHR FLEGKIKPHL MSQEVPEWD	KQPVKVLVGA	
Pdi_Rat	KPESDELTAE K..ITQFCHH FLEGKIKPHL MSQELPEDWD	KQPVKVLVGA	
Pdi_Bovin	KPESDELTAE K..ITEFCHR FLEGKIKPHL MSQELPDDWD	KQPVKVLVGA	
Pdi_Human	KPESEELTAE R..ITEFCHR FLEGKIKPHL MSQERAGWD	KQPVKVPVGA	
Pdi_Rabit	KPESDELTAE G..ITEFCQR FLEGKIKPHL MSQELPEDWD	RQPVKVLVGA	
Pdi_Chick	KPESDDLTAD K..IKEFCNK FLEGKIKPHL MSQDLPEDWD	KQPVKVLVGA	
Pdi_Yeast	DELSDKIVLE SKAIESLVKD FLKGDASPIV KSQEIFENQD	S.SVFQLVGA	
	401		450
Pdi_Mouse	NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	KDHENIIIAK	
Pdi_Rat	NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	KDHENIVIAK	
Pdi_Bovin	NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	KDHENIVIAK	
Pdi_Human	NFEDVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	KDHENIVIAK	
Pdi_Rabit	NFEEVAFDEK KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	KEHQDIVIAK	
Pdi_Chick	NFEEVAFDEN KNVFVEFYAP WCGHCKQLAP IWDKLGETY.	RDHENIVIAK	
Pdi_Yeast	NHDEIVNDPK KDVLVLYYAP WCGHCKRLAP TYQELADTYA	NATSDVLIK	
	451		500
Pdi_Mouse	MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD	GFKKFLES GG	
Pdi_Rat	MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD	GFKKFLES GG	
Pdi_Bovin	MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD	GFKKFLES GG	
Pdi_Human	MDSTANEVEA VKVHSFPTLK FFPASADRTV IDYNGERTLD	GFKKFLES GG	
Pdi_Rabit	MDSTANEVEA VKVHSFPTLK FFPAGPGRTV IDYNGERTLD	GFKKFLES GG	
Pdi_Chick	MDSTANEVEA VKIHSFPTLK FFPAGSGRNV IDYNGERTLE	GFKKFLES GG	
Pdi_Yeast	LDHTENDVRG VVIEGYPTIV LYPGGKKSSES VVYQGSRLD	SLFDFIKENG	
	501		538
Pdi_Mouse	QDGAGDDEDL .DLEE..ALE PDMEE..DDD QKAVKDEL		
Pdi_Rat	QDGAGDNDL .DLEE..ALE PDMEE..DDD QKAVKDEL		
Pdi_Bovin	QDGAGDDDDL EDLEE..AEE PDLEE..DDD QKAVKDEL		
Pdi_Human	QDGAGDDDDL EDLEE..AEE PDMEE..DDD QKAVKDEL		
Pdi_Rabit	QDGAGDEDGL EDLEE..AEE PDLEE..DDD QKAVRDEL		
Pdi_Chick	QDGAAADDDL EDLET..DEE TDLEEGDDDE QKIQKDEL		
Pdi_Yeast	HFDVDGKALY EEAQEKAEE ADADAELADE EDAIHDEL		

Table 2:

Alfalfa	M-AKNVAIFG	LLFSLLLLVP	SQIFA-----	-----EES	STDAKE-----
Oryzae	MRTFAPWIL-	--SLLGASA-	--VAS-----	-----AADA	TAEAPS-----
Yeast	MKFSAGAVLS	WSSLLLLASS-	--VFA-----	-----QQEA	VAPEDS-----
Bovine	M-LRRA-LLC	--LALTALF-	--RAG-----	-----AGA	PDEEDH-----
Rat	M-LSRA-LLC	--LALAWAA-	--RVG-----	-----ADA	LEEEDN-----
Mouse	MKLRKAWLLV	LLLALTQLLA	AASAGDAQED	TSDTENATEE	EEEEDDDDLE
			---FVL---	-----	-----
			---DVV---	-----	-----
			---AVV---	-----	-----
			---VL---	-----	-----
			---VL---	-----	-----
	VKEENGWVVL	NDGNFDNFVA	DKDVTLLLEFY	APWCGHCKQF	APEYEKIAST
			---TLDNT-	-----	-----
			---SLTGD-	-----	-----
			---KLATD-	-----	-----
			---VLHKG-	-----	-----
			---VLKKS-	-----	-----
	LKDNDPPIAV	AKIDATSASM	LASKFDVSGY	PTIKILKKGQ	AVDYDGSRTQ
			---NF	HDTVKKHDFI	VVEFYAPWCG
			---TF	ETFVKEHDLV	LAEFFAPWCG
			---SF	NEYIQSHDLV	LAEFFAPWCG
			---NF	DEALAAHKYL	LVEFYAPWCG
			---NF	AEPAAHNYLL	VEFY-APWCG
	EEIVAKVREV	SQPDWTPPPE	VTLSLTKDNF	DDVVNNADII	LVEFYAPWCG
	HCKKLAPEYE	KAASILSTHE	PPVVLAKVDA	NEEHNKDLAS	ENDVKGFPTI
	HCKALAPKYE	QAATELKEKN	IPL--VKVDC	TEEEA--LCR	DQGVGYPTL
	HCKNMAPEYV	KAAETLVEKN	ITL--AQIDC	TENQD--LCM	EHNIPIGFPSL
	HCKALAPEYA	KAAGKLKAEG	SEIRLAKVDA	TEESD--LAQ	QYGVRGYPTI
	HCKALAPEYA	KAAAKLKAEG	SEIRLAKVDA	TEESD--LAQ	QYGVRGYPTI
	HCKKLAPEYE	KAAKELSKRS	PPIPLAKVDA	TEQTD--LAK	RFDVSGYPTL
	KIFRNGG-KN	IQEYKGPREA	EGIVEYLKKQ	SGPAS-TEIK	SADDATAFVG
	KIFRGLDAVK	P--YQARQT	EAIVSYMVKQ	SLPAV-SPVT	PENLEE-IKT
	KIFKNRDVNN	SIDYEGPRTA	EAIVQFMIKQ	SQPAV-AVVA	DLPAYL-ANE
	KFFKNGDTAS	PKEYTAGREA	DDIVNWLKKR	TGPAA-STLS	DGAAAEALVE
	KFFKNGDTAS	PKEYTAGREA	DDIVNWLKKR	TGPAA-TTLS	DTAAAEALVD
	KIFRKG---R	PFDYNGPREK	YGIVDYMIEQ	SGPPSKEILT	LKQVQEFLKD
	DNKVIVGVGF	PKFSGEEYDN	FIALAEKLRS	DYDFAHTLNA	KHLPKGDSSV
	MDKIVVIGYI	ASDDQTANDI	FTTFAESQRD	NYLFAATSDA	SI--AKAEGV
	TFVTPVIVQS	GKIDADFNAT	FYSMANKHFN	DYDFVSAENA	DD--DFKLSI
	SSEVAVIGFF	KDMESDSAKQ	FLLAAEVI-D	DIPFGITSNS	DV--FSKYQL
	SSEVTVIGFF	KDAGSDSAKQ	FLLAAEAV-D	DIPFGITSNS	DV--FSKYQL
	GDDVVIIGLF	QGDGDPAYLQ	YQDAANNLRE	DYKFHHTFSP	EIAKFLKVS

table 2 (continued)

SGPVVRLFKP FDELFVDS-- -KDFNVEALE KFIEESSTPI VTVFNNEPSN  
 KQPSIVLYKD FDEKKATYDG EIEQDALLSW VKTASTPLVG ELGPETYSGY  
 YLPSAM--DE PVVYNGKKAD IADADVFEKW LQVEALPYFG EIDGSVFAQY  
 DKDGVVLFKK FD---EGR-- -NNFEGEVTK EKLLDFIKHN QLPLVIEFTE  
 DKDGVVLFKK FD---EGR-- -NNFEGEITK EKLLDFIKHN QLPLVIEFTE  
 GKLVLTTHPEK FQSKYEPRFH VMDVQGSTEA SAIKDYVVKH ALPLVGHKRT

HPFVVVKFFNS PNAKAMLFIN FTTEGAESFK TKYHEVAEQY KQQGV-SFLV  
 ITAGIPLAYI FAETKEEREQ FTEEFKFIAE KHKGSINIIVT IDAKLYGAHA  
 VESGLPLGYL FYNDEEELEE YKPLFTELAK KNRGLMNFVS IDARKFGRHA  
 QTAPKIFGGE IKTHILLFLP KSVSDYEGKL SNFKKAAESF KGKILFIFID  
 QTAPKIFGGE IKTHILLFLP KSVSDYDGKL SNFKKAAEGF KGKILFIFID  
 SNDAKRYSKR PLVVVYYSVD FSFDYRAATQ FWRNKVLEVA KDFPEYTFAI

GDVESSQGAF QYFGLKEEQV PLI--IIQHN DGKKFFKPN- --LELDQLPT  
 GNLNLDPSKF PAFAIQDPEK NAKY----- --PYDQSKE- --VKAKDIGK  
 GNLNMK-EQF PLFAIHDMTE DLKYGLPQLS EEAFFDELSK IVLESKAIES  
 SDHTDNQRIL EFFGLKKEEC PAVR-LITTLE EEMTKYKPES DELTAEKITE  
 SDHTDNQRIL EFFGLKKEEC PAVR-LITTLE EEMTKYKPES DELTAEKITQ  
 ADEEDYATEV KDLGL-SESG EDVN-AAILD ESGKKFAMEP EEFDSDTLRE

WLKAYKDGKV EPPVKSEPIP ETNN-EPVKV VVGQTLEDV FKSQKQNVLIE  
 FIQDVLDDKV EPSIKSEAI P ETQE-GPVTV VVAHSYKDLV LDNEKDVLLE  
 LVKDFLKGDA SPIVKSQEIF ENQD-SSVFQ LVGKNHDEIV NDPKKDVLVL  
 FCHRFLEGKI KPHLMSQELP DDWDKQPVKV LVGKNFEEVA FDEKKNVFVE  
 FCHRFLEGKI KPHLMSQELP EDWDKQPVKV LVGKNFEEVA FDEKKNVFVE  
 FVTAFKKGKL KPVIKSQPVP KN-NKGPVKV VVGKTFDAIV MDPKKDVLIE

FYAPWCGHCK QLAPILDEVA VSFQS-DADV VIAKL DATAN DIPTDTFDVQ  
 FYAPWCGHCK ALAPKYEELA SLYKD-IPEV TIAKIDATAN DV--PD-SIT  
 YYAPWCGHCK RLAPTYQELA DTYANATSDV LIAKLDHTEN DV--RGVVIE  
 FYAPWCGHCK QLAPIWDKLG ETYKD-HENI VIAKMDSTAN EV--EAVKVH  
 FYAPWCGHCK QLAPIWDKLG ETYKD-HENI VIAKMDSTAN EV--EAVKVH  
 FYAPWCGHCK QLEPIYTSLG KKYKG-QKDL VIAKMDATAN DITNDQYKVE

GYPTLYFRSA SGK--LSQYD GGRTKEDIIE FIE-----K NKDKTGAHQ  
 GFPTIKLFAA GAKDSPVEYE GSRTVEDLAN FVK-----E NGKHKVDAL  
 GYPTIVLYPG GKSESVVYQ GSRSLDSLFD FIK-----E NGHFDVDGKA  
 SFPTLKFFPA SADRTVIDYN GERTLDGFKK FLESGGQDGA GDDDDLEDLE  
 SFPTLKFFPA SADRTVIDYN GERTLDGFKK FLESGRQDGA GDNDLDLEE  
 GFPTIYFAPS GDKKNPI--- -----K F-----E GGNRDLEHLS

EVEQPKAAAQ PE----- ----- AEQPKDEL  
 VDPKKEQESG DATETRAASD ETETPAATSD DKSEHDEL  
 LYEEAQEKAA EEAEADAEE ADADAELADE EDAIHDEL  
 EAEPPDLEED DD----- ----- QKAVKDEL  
 ALE-PDMEED DD----- ----- QKAVKDEL  
 KF--ID-EHA TK----- ----- RSRTKEEL

**PATENT CLAIMS**

1. A method of producing a protein disulfide redox agent, comprising
- i) cloning a DNA sequence coding for said protein disulfide redox agent from a donor cell,
  - 5 ii) making a DNA construct wherein said DNA sequence is under control of regulatory elements,
  - iii) introducing said DNA construct into a host cell,
  - iv) growing said host cell under conditions conducive to the production of the protein disulfide redox agent, and
  - 10 v) recovering and purifying said protein disulfide redox agent.
2. The method according to claim 1, wherein said protein disulfide redox agent is secreted into the medium.
3. The method according to claim 1 to 2, wherein said DNA construct is introduced into a host cell of a species different from the donor cell.
- 15 4. The method according to claim 1 to 3, in which the protein disulfide redox agent is expressed in the form of a proenzyme and the cell is cultured in the presence of a proteolytic enzyme capable of converting the proenzyme of the protein disulfide redox agent into a mature enzyme.
5. The method according to claim 1 to 4, wherein both said donor and host cells  
20 are microbial.
6. The method according to claim 1 to 5, wherein said donor and host cell are either a bacterial cell or a fungal cell.
7. The method according to claim 6, wherein both said donor and host cells are bacterial.

8.The method according to claim 7, wherein one of said bacterial cells is gram-positive and one is gram-negative.

9.The method according to claim 6, wherein both said donor and host cells are fungal.

5 10.The method according to claim 6, wherein one of said microbial cells is bacterial and one is fungal cell.

11.The method according to claim 5 to 10, in which the bacterial cell is a cell of a gram-positive bacterium, e.g. of the genus *Bacillus* or *Streptomyces* or a cell of a gram-negative bacterium, e.g. of the genus *Escherichia*, and the fungal cell is  
10 a yeast cell, e.g. of the genus *Saccharomyces*, or a cell of a filamentous fungus, e.g. of the genus *Aspergillus* or *Fusarium*.

12.The method according to claim 11, wherein said *Escherichia* is *E. coli*.

13.The method according to claim 11, wherein said *Aspergillus* is *Aspergillus niger*, *Aspergillus oryzae*, or *Aspergillus nidulans*.

15 14.The method according to claim 11, wherein said *Bacillus* is *Bacillus licheniformis*, *Bacillus lentus*, or *Bacillus subtilis*.

15.A protein disulfide redox agent product produced by any of the methods of claim 1 to 14.

20 16.The product according to claim 15, wherein said protein has a prolonged N-terminal.

17.The product according to claim 16, wherein said prolongation comprises an Alanine in the N-terminal.

18.The product according to claim 17, wherein said protein is DsbA or a thioredoxin.

19.A composition of matter comprising (i) a protein disulfide redox agent produced according to claim 1-14, optionally (ii) at least a redox partner, and  
5 optionally (iii) one or more other enzymes.

20.The composition of claim 19, wherein said protein disulfide redox agent (i) is a protein disulfide isomerase, -oxidase, -reductase, -oxidoreductase, a thioredoxin, a sulfhydryl oxidase, -oxidoreductase, -reductase, or -transferase, capable of catalyzing the reduction/oxidation of protein disulfide linkages.

10 21.The composition of claim 20, comprising a product of claim  
15 to 18.

22.The composition of claim 19, wherein said redox partner (ii) is an organic or inorganic reductant.

23.The composition of claim 22, wherein said organic reductant is selected from  
15 the group comprising glutathione, L-cysteine, dithiothreitol, 2-mercaptoethanol, thioglycolic acid, L-cysteine ethylester,  $\beta$ -mercaptoethylamine, mercaptosuccinic acid,  $\beta$ -mercaptopropionic acid, dimercapto adipic acid, thiomalic acid, thioglycolamides, glycol thioglycolate, glycerol thioglycolate, thiolactic acid and salts thereof.

20 24.The composition of claim 23, wherein said inorganic reductant is selected from the group comprising sulfite and bisulfite.

25.The composition of any of the preceding claims, in which said other enzyme (iii) is a protease, a lipase, an amylase, a transglutaminase, or another protein disulfide redox agent.



26.A process for treating scleroproteins which comprises applying the composition of claim 19 to 25 to the scleroprotein.

27.The process of claim 26, wherein said scleroprotein is human hair or animal hair.

5 28.The process of claim 27, wherein said process involves waving, straightening, degrading or softening of said hair.

29.A process for the cleaning of fabrics involving the application of the composition of claim 19 to 25 to said fabrics.

30.The process of claim 29 also involving treatment with a detergent.

10 31.A process for thickening and/or gelation of food involving application of the composition of claim 19 to 25 to the food.

32.A process for the dissolution of lung gels involving application of the composition of claim 19 to 25 to the lungs.

15 33.A process for the alleviation of certain eye conditions involving application of the composition of claim 19 to 25 to the eyes.

34. A DNA construct encoding a protein disulfide redox agent product according to claim 15 to 18.

35.The DNA construct according to claim 34, wherein said DNA sequence is under control of regulatory elements.

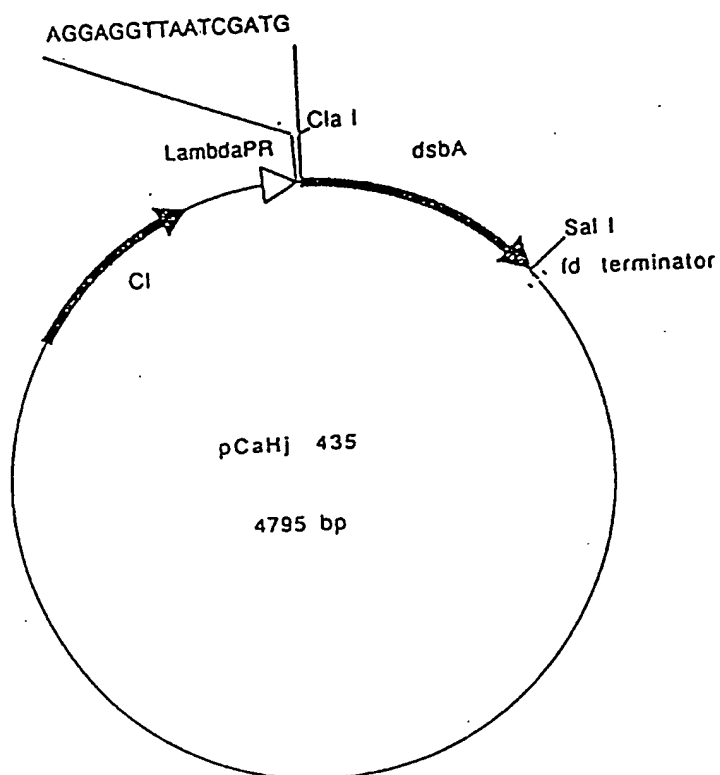
20 36.An expression vector comprising a DNA construct according to claim 34 to 35.

37. The vector of claim 36, wherein said DNA construct is operably linked to a promoter sequence and optionally to a sequence encoding a secretion signal.

38. A cell comprising a vector according to claim 36 to 37.

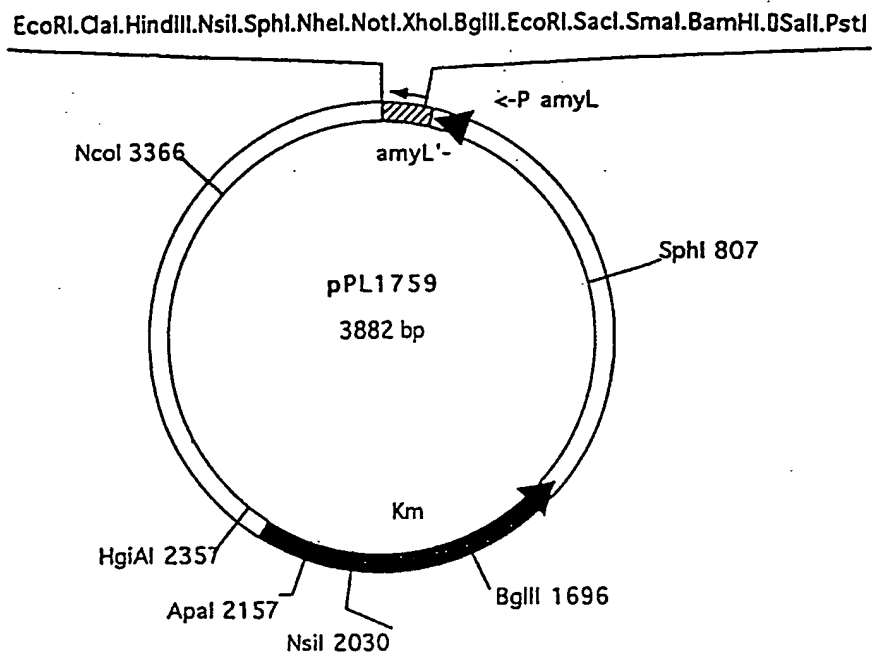
1/3

Figure 1:



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Figure 2:



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Figure 3:

